



IN THE UNITED STATES PATENT OFFICE

APPLICANT: Shih-Chieh Hung
APPLICATION No.: 09/761,893
FILING DATE: 01/17/2001

ART UNIT: 1636
EXAMINER: Jennifer Dunston
TITLE: Method of isolating
mesenchymal stem cells

DECLARATION OF SHIH-CHIEH HUNG, PH.D.

Dear Sir,

Shih-Chieh Hung, Ph. D., hereby declare as follows:

1. I am a professor of National Yang-Ming University and Visiting Staff of Department of Orthopedics Taipei veterans General Hospital. I am the first inventor of this application, who has been conducting research on mesenchymal stem cells for 20 years.
2. I believe that this application created an unexpected result or significant improvement at the time of this invention. Especially, the cell populations having greater than 98% of human MSCs could be obtained in accordance with the method of the invention, and such isolated MSCs can proliferate without differentiation and reach confluence even after 12 passages, which solved part of the long-felt need problems of human MSCs.
3. The working example on page 5 of this application in Chinese version, attached, did mention the upper plate with pore with 0.4 to 16 microns in diameter.
4. I solemnly declare that all statements made herein of my own knowledge are true and that all statement made on information and belief are believe to be true.

Dated: 0429.2011

By: Shih-Chieh Hung
Shih-Chieh Hung

五、發明說明 (10)

) 的肝素處理而抑制血球凝固。將處理過的骨髓與等量的磷酸鹽緩衝溶液 (PBS) 混合，並在室溫下，以 900 xg 離心 10 分鐘。將清洗的細胞再懸浮於 PBS 中，至最終體積為 5 毫升，並且覆於等體積之 1.073 克/毫升的 Percoll 溶液上，以 900 xg 離心 30 分鐘。收集在界面之單核細胞層。將溶於含有 10% 胎牛血清之 DMEM-LG (含 1 克/公升葡萄糖之 Dulbecco's 改質的 Eagle's 培養液; Life Technologies) 培養液中，以及抗生素 (青黴素 100 單位/毫升，鏈黴素 100 微克/毫升) 的 Percoll 分離或未分離之骨髓細胞，以 10^6 /平方公分的密度接種至培養裝置的上層。此培養裝置包括上下兩層，上層具有大約 0.4 到 16 微米範圍孔徑之膜板，下層為一塑膠培養皿。將細胞培養物維持在 37°C，5% CO₂ 中，第一次接種後 7 天更換培養液，以後每隔 4 天更換一次。

實施例 2：從骨髓細胞中分離並擴展間質細胞

勻稱的纖維芽細胞選植株可在大約接種後 7 天觀察到。藉由更換培養液而移除造血幹細胞及未附著的細胞。當細胞長滿時，以 0.25% 的胰蛋白酶-EDTA 將其回收，再以 4×10^3 - 10^4 /平方公分的密度，接種至培養盤中擴展間質細胞。將所得到之擴展的細胞用於測試複製更新的能力，以及對於標幟蛋白質之特定反應。

實施例 3：流體細胞測量分析及定性

流體細胞測量分析是用於了解間質細胞的均質性並定性其表面抗原。將分離且擴展的間質細胞，藉由特定表面

